RESEARCHREPORT
SWINE HEALTH
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Title: Optimization of the PRRSV antibody ELISA for use in oral fluid-based surveillance – **NPB #08-262**

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ABSTRACT

Already a proven technology in human diagnostic medicine, oral fluid-based testing could facilitate monitoring of disease in animal populations. Availability of disease data could provide for (1) cheaper methods of surveillance; (2) critically timed and targeted interventions; (3) "real time" evaluations of interventions; and (4) accurate estimates of the impact of specific pathogens on pig health and productivity. Therefore, the goal of this research was to provide pork producers an easy, cost-effective method to detect and monitor PRRSV circulation in swine populations using an antibody assay optimized and validated for oral fluid samples. The results of this experiment showed that a commercial PRRS ELISA could be optimized to detect anti-PRRSV antibody in oral fluid samples. Subsequent to this work, the manufacturer has developed a next generation assay reported to provide improved performance, e.g., fewer false positive results. The results of this study justify evaluation of the PRRS 3X ELISA for detection of anti-PRRSV antibody in oral fluids.

INTRODUCTION

Diagnostic components in oral fluid The fluid in the oral cavity consists of saliva and transudates originating from the circulatory system. The process of "passive transudation" was first demonstrated by intravenously injecting fluorescein dye into the hind leg of dogs (n = 6) and recording fluorescence on filter paper strips collected within and at the gingival crevice (Brill and Krasse, 1958). In these experiments, the dye appeared at the gingival crevice within 30 seconds after injection. Thus, diagnostic targets in oral fluids may be produced locally or originate from the circulatory system.

Viruses in oral fluids Wills et al. (1997) reported the isolation of PRRSV from oral fluids collected from individual PRRSV-infected pigs through the last sampling point (42 DPI). More recently, Prickett et al. (2008a,b) demonstrated the validity and utility of pen-based oral fluid samples for the detection or PRRSV and PCV2 by PCR. Both of these viruses were readily detected in oral fluid samples and the results correlated well with results based on matched serum samples. In addition, vesicular stomatitis virus (Stallnecht et al., 1999) foot-and-mouth disease virus (Eblé et al., 2004), influenza virus (Irwin et al., 2009), and classical swine fever virus (CC Chang, personal communication) have been detected in porcine oral fluids.

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Antibody in oral fluids The first report of antibodies in oral fluid from pigs was published in 1976 when Corthier (1976) reported that intranasal vaccination of pigs with the Thiverval strain of classical swine fever virus (CSFV) resulted in detectable antibody in pharyngeal secretions. They subsequently inoculated pigs via intranasal and intramuscular routes and measured the antibody response in serum and oral fluid (Corthier and Aynaud, 1977). Strong responses were measured in both sample types, indicating both systemic and local immune responses. The strongest local response (in oral fluid) was observed after intranasal inoculation at the highest dose used.

DeBuysscher and Dubois (1978) inoculated pigs with E. coli strain 1261 via oral or Thiry-Vella loop routes and examined the submandibular and sublingual salivary glands post mortem by staining for anti-E.coli plasma cells of the IgA, IgM, and IgG isotypes. They reported IgA-secreting plasma cells to be most numerous followed by approximately equal numbers of IgM- and IgG-secreting cells, but found no significant differences in the number and isotype of plasma cells in submandibular and sublingual salivary glands between pigs orally or Thiry-Vella loop inoculated pigs.

A few years later DeBuysscher and Berman (1980) performed essentially the same experiment with transmissible gastroenteritis virus (TGEV). They observed substantial increases in the number of IgA-secreting cells in salivary glands, followed by IgM-secreting cells, and a small increase in the number of IgG-secreting cells in both orally and Thiry-Vella loop inoculated pigs. These findings supported the conclusion that corollary increases in antigen-specific plasma cells in intestinal mucosa and distant secretory tissues, i.e., salivary glands, resulted from the secretory component receptor-conveyor mechanism.

Loftager et al. (1993) collected samples from pigs intranasally inoculated with *Actinobacillus pleuropneumoniae* (APP) and pigs naturally infected with APP. IgA concentrations in oral fluid and serum collected over time were measured using a whole-cell ELISA. IgA was detectable in oral fluid before it appeared in serum and declined more rapidly than serum IgA or IgG. These investigators concluded that oral fluid IgA detection could serve as a practical method to screen for early infection with APP.

Preliminary observations justifying this research Preliminary evaluation of the use of the IDEXX PRRS ELISA for detection of anti-PRRSV antibodies in oral fluids was performed using oral fluid samples (n = 66) from a previous PRRSV experiment. Modifications of the standard PRRS ELISA protocol included (1) samples were heat inactivated for 30 minute at 56°C to prevent non-specific NHC well reactions; (2) each sample was tested at 3 dilutions (undiluted, 1:3, 1:5; (3) plate positive controls were run at 3 dilutions: undiluted, 1:2, 1:4. Samples were incubated overnight at 4°C. Plates were brought to room temperature in the morning and the controls were added. The remainder of the assay was performed according to the manufactures instructions. Receiver operator characteristic curves (ROC) were used to assess the performance of the assay at all combinations of sample and control dilutions and estimate S/P cut-offs.
The assay performed best at a sample dilution of 1:3 and positive control dilution of 1:5. At an S/P cut-off value of ≥ 0.25, the diagnostic sensitivity (Se) was 84% and diagnostic specificity (Sp) was 100%. These results supported the premise that the commercial PRRS ELISA could be modified for use with oral fluid samples. This modification would allow monitoring PRRSV circulation at a much lower cost that possible with PCR-based assays.

PROJECT OBJECTIVES

The broad goal of the proposed research was to provide pork producers an easy, cost-effective method to detect and monitor PRRSV circulation in swine populations using an antibody assay optimized and validated for oral fluid samples. The specific objective of the study was to optimize the PRRS ELISA for detection of antibodies in oral fluid samples: (1) determine the optimal oral fluid sample and positive control dilutions; (2) determine

the optimal sample incubation time; (3) establish a cut-off value for the modified ELISA protocol; and (4) estimate diagnostic performance of the assay.

MATERIALS AND METHODS

5.1 Experimental Design

Taguchi Design of Experiments methodology was used to design a L-9 orthogonal array-based series of experiments for the optimization of the oral fluid PRRSV ELISA. The project was completed in step-wise phases. The objective of Phase (1) was to determine the optimum operating condition for maximal antigen (Ag) well absorbance and minimum normal host (NHC) cell well absorbance. Phase (2) was designed to optimize the concentration of antibody in the kit positive control reagent and the optimum incubation time for the kit positive control reagent. Phase (3) was designed to determine the optimized S/P ratio cut-off value for oral fluid using the modified protocol to test an set of longitudinal oral fluid samples of know status using receiver operator characteristic curve analysis. Phase (4) was designed to test a longitudinal set of oral fluid samples from the field using the optimized protocol.

5.2 Assay conditions to optimize Ag well OD and minimize NHC OD

Sample selection: To be representative of the general swine population 14 of the 16 samples used in Phase 1were randomly selected from a set of longitudinal oral fluid samples collected from several populations of growing pigs across the Midwest. These populations were comprised of finisher pigs of varying sex, ages (maternal antibody levels), disease status, vaccination status, and geographic locations. The 14 samples were randomly drawn from samples collected longitudinally in 10 populations (barns) of pigs in the Midwest. Samples 1 – 10 were drawn so that one sample came from each of the 10 populations, and 4 of the populations were randomly drawn to provide an additional randomly drawn sample (samples 11-14). Samples 15 and 16 were oral fluid samples of known status collected from an experimental PRRSV inoculation. Sample 15 was collected from a pen of 8-week old mixed gender pigs on 13 DPI. Sample 16 was a negative control pen of 4-week old pigs collected on 7 DPI.

Selection of factors and levels: The design of the optimization phase of the experiment resembled a Taguchi design for process improvement. JMP statistical software (SAS Institute Inc., Cary, NC USA) was used to design an inner orthogonal array for the designation of the combinations of factors (variables) and levels to be compared. With this method, the user determines what factors at what levels are to be included, the software designs the array. This differs from a full factorial design in that not all combinations of factors and levels are compared. From previous data (not shown) the expected optimum combinations of levels of the factors to be investigated was approximately known. To further refine the optimization, one

Table 1. Factor and level descriptions

Factors	Level 1	Level 2	Level 3
Sample dilution	1:2	1:3	1:4
Incubation time (hours)	4	6	16
Heat treatment (minutes)	0	10	30

Table 2. L-9 orthogonal array

Experiment	Level of Factor 1	Level of Factor 2	Level of Factor 3
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

level above and one level below the expected optimum level of each factor were included in the experiment. Table 1 summarizes the factors and levels tested.

Three factors were selected for optimization and were tested at 3 levels each: (1) sample dilutions of 1:2, 1:3, 1:4; (2) incubation times of 4 hours, 6 hours, and 16 hours; and (3) heat treatment times of 0 minutes, 10 minutes, and 30 minutes. Table 2 displays the L-9 orthogonal array generated by the JMP software.

Protocol: At time -2 hours, the 16 samples were thawed at room temperature under a laminar flow hood for 30 minutes, while the appropriate volume of diluent was pipette into 96-well dilution plates. At the end of the 30 min. thaw, the samples were divided into 3 1ml aliquots, each destined for a different duration of heat treatment. While the 30 min. heat treated samples were in the 56°C water bath, the 0 minute heat treated samples were pipetted into the appropriate wells of the dilution plates containing diluent. The 30 minutes heat-treated samples were then pippeted into the dilution plates while the remaining aliquot was heat treated for 10 minutes and then pipetted into the dilution plates. There were 3 dilution plates in total, 1 for each of the 3 incubation periods to be investigated. Each dilution plate contained enough sample and diluent for each sample to be run in triplicate. At time 0, each of the 3 dilution plates were transferred onto ELISA plates in triplicate according to plate maps, so that there were 3 plates for each incubation time, each representing the entire orthogonal array previously described. The plates requiring incubation for 4 and 6 hours were incubated in a temperature controlled environmental chamber set at 23°C and the 16 hour incubation was performed in a controlled 4°C refrigerator/incubator. At time 4 hours, the 4 hours plates were removed from the incubator and the assay was completed according to the manufactures instructions, similarly to the 6 hour plates at time 6 hours. The 16 hour plates were removed from the 4°C refrigerator/incubator at time 15.5 hours and placed in the 23°C for the last 0.5 hours before completion of the assay. All plates were read at 650nm on a Biotek EL808 plate reader using Biotek Gen5 software.

Analysis: The analysis was performed with JMP 6.0.0 software using the Taguchi Design of Experiments platform and the Fit Model (standard least squares) analysis. The data was entered as 48 individual data points for each experiment, i.e., 16 samples in total multiplied by 3 replicates of each sample. The statistical analysis was performed 3 times, once each for the O.D. results of the antigen coated well (Ag), the normal host cell well (NHC), and the arithmetic difference between the Ag and NHC wells. Analysis of variance was used to assess the respective contribution of each factor. The prediction profiler tool in JMP was used to determine the optimum level of each factor based on sample means for each of the O.D. results (Ag, NHC, and Ag-NHC). Selection of optimum levels was based on maximizing the Ag O.D. and minimizing the NHC O.D means.

5.3 *Selection of positive control dilution, cut-off selection, and performance evaluation*

Positive control dilution selection: Prior to testing the experimental samples in phase 2, a pool of kit positive control reagent was 5-fold serially-diluted from undiluted to a 1:35 dilution and assayed on ELISA plates and the corrected absorbance was recorded at 30 min., 2 h, 4 h, 8 h, 12 h, and 16 h.

Sample selection for cut-off selection and performance evaluation: A set of longitudinal samples described in Prickett et al., 2008 were used to evaluate performance and determine an appropriate cut-off point for the optimized ELISA. In brief, the sample set was comprised of 2 to 3 samples per pen per week from a 9-week PRRSV inoculation study. The sample set included 156 samples from PRRSV-inoculate animals and 60 samples from negative controls. The samples were blindly labeled and randomized prior to testing.

Assay protocol: The PRRS ELISA for detection of antibodies in oral fluid was performed according to the manufactures protocol under the conditions determined in phase 1 of this experiment. In brief, samples were run at a dilution of 1:2 after being heat treated at 56°C for 10 minutes with an incubation period of 16 hours at 4°C. Four positive control dilutions were run on each plate: undiluted, 1:4, 1:5, and 1:6. Positive controls were diluted in the diluents provided by the manufacturer. S/P values were calculated for each sample using each of the 4 positive control dilutions. The positive control dilution resulting in the optimized S/P response was identified, i.e. the dilution resulting in the appropriate magnitude of difference between positive and negative samples.

Cut-off selection and performance evaluation: Receiver operator characteristic curve (ROC) analysis was used to determine the ROC optimized cut-off value and the corresponding performance (diagnostic sensitivity and specificity).

RESULTS

6.1 Assay conditions to optimize Ag well OD and minimize NHC OD

Based on the prediction profiler in the output of an ANOVA model run in JMP, a 1:2 sample dilution was determined to provide the maximum antigen well absorbance and minimum NHC well absorbance. Heat inactivation (10 minutes at 56°C) produced optimum results. Similarly, the optimum incubation time was calculated to be 16h at 4°C.

Positive control incubation data indicated (Figure 1) that maximum absorbance was achieved by 12 h and a 1:10 dilution resulted in the desired level of reactivity as determined by previous work (data not shown).

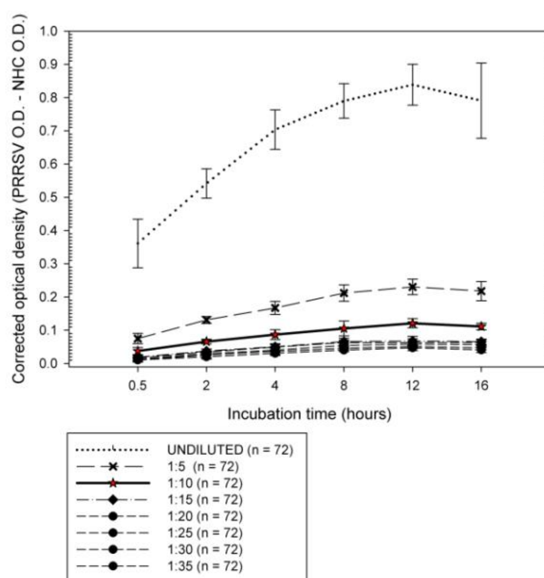

Figure 1. Positive control response by dilution and incubation time

Positive control dilution, cut-off selection, and performance evaluation

The results of the longitudinal study are displayed in Figure 2. Table 3 displays the potential cut-off values and corresponding diagnostic performance. Optimum results were obtained using a positive control dilution of 1:6.

Table 3. Positive control dilution and assay performance.

	ROC optimized performance					Surveillance optimized performance[#]				
Pos. Dil.	*C_O	Se_O	95% CI Se_O	Sp_O	95% CI Sp_O	#C_S	Se_S	95% CI Se_S	Sp_S	95% CI Sp_S
UN	>0.05	87%	81 – 92%	93%	82 – 98%	>0.07	73%	65 – 80%	100%	94 – 100%
1:4	>0.18	85%	78 – 90%	93%	84 – 98%	>0.24	72%	65 – 79%	100%	94 – 100%
1:5	>0.21	89%	82 – 93%	92%	82 – 97%	>0.28	74%	67 – 81%	100%	94 – 100%
1:6	>0.27	85%	79 – 90%	93%	84 – 98%	>0.36	76%	68 – 82%	100%	94 – 100%
1:7	>0.28	88%	82 – 93%	93%	84 – 98%	>0.38	76%	67 – 83%	100%	94 – 100%

*ROC optimized cut-off is defined as the cut-off that cumulatively results in the best diagnostic performance.
[#] Surveillance optimized cut-off is the cut-off resulting in 100% specificity.

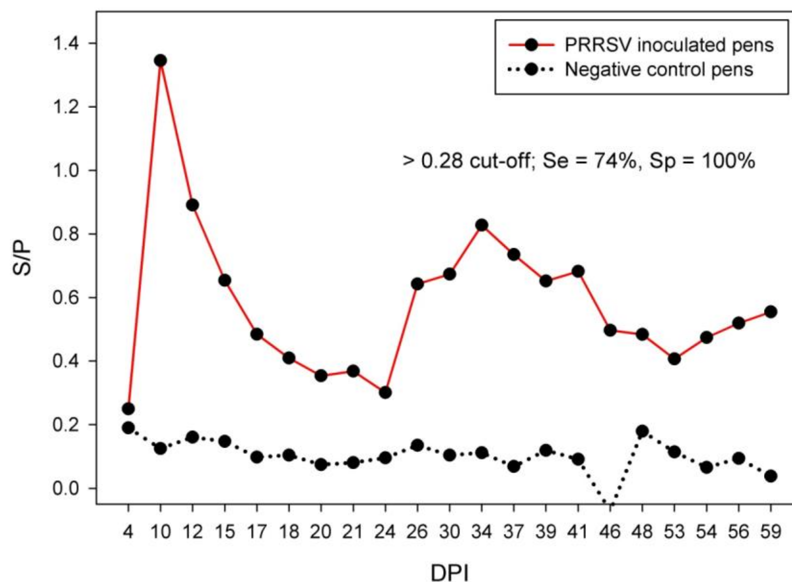

Figure 2. ELISA results in oral fluid collected over time post inoculation

DISCUSSION

The results of this experiment showed that the IDEXX ELISA could detect anti-PRRSV antibody in oral fluid samples. Subsequent to this work, IDEXX has developed a new PRRSV ELISA (X3) reported to provide improved performance, e.g., fewer false positive results. The results of this study justify evaluation of the PRRS 3X ELISA for detection of anti-PRRSV antibody in oral fluids.

LAY INTERPRETATION

PRRSV, *M. hyopneumoniae,* influenza, PCV2, and other endemic pathogens continue to cause significant economic losses to U.S. swine producers. In part, the disease *status quo* is maintained by the absence of timely information on the circulation of pathogens. Already a proven technology in human diagnostic medicine, oral fluid-based testing could facilitate monitoring of disease in animal populations. Availability of disease data would facilitate (1) appropriately timed and targeted interventions; (2) "real time" evaluations of interventions; and (3) accurate estimates of the impact of specific pathogens on pig health and productivity.

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